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1. Your reference — Cp11c7gb

2. Patent application number *(The Patent Office will)* — **9916703.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

1 ANTONIO ALCAMI, of 46 Hauxton Road, Little Shelford, Cambridge
2 STACEY EFSTATHIOU, of 18 Norwich Street, Cambridge, and
3 CHRISTOPHER MARC PARRY, of 37 Langham Road, Cambridge

Patents ADP number *(if you know it)*

1 7702590001
2 7702608001
3 7702616001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

VIRAL PROTEIN BINDING COMPOSITIONS and METHODS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Mr T C Stanclyffe
Cantab Pharmaceuticals Research Limited
310 Cambridge Science Park,
Milton Road, Cambridge CB4 0WG

Patents ADP number *(if you know it)* 7639123001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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Yes

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Continuation sheets of this form	
Description	11
Claim(s)	—
Abstract	— 1
Drawing(s)	2

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	—
Request for preliminary examination and search *(Patents Form 9/77)*	—
Request for substantive examination *(Patents Form 10/77)*	—
Any other documents *(please specify)*	—

11. I/We request the grant of a patent on the basis of this application.

Signature TCStancliffe Date 16/7/99

12. Name and daytime telephone number of person to contact in the United Kingdom TCSTANCLIFFE 01223 436508

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Viral protein binding compositions and methods

Field of the Invention:

This invention relates to the use of viral proteins and analogues thereof as binding partners for immune system components and analogues thereof, and to related compositions and methods, for example pharmaceutical compositions and methods, and detection or assay reagents and kits and methods.

Background of the Invention:

Among known herpesvirus proteins is a protein encoded by gene M3 of murine gammaherpesvirus 68 (MHV68) (V van Berkel et al: J Virol 73(5) (1999) pp 4524-4529).

Protein M3 of MHV68 has been reported to be a secreted protein. It has been suggested that this protein may modulate the host immune response to infection by the virus.

The present invention arises from a new finding of particular binding properties of M3 protein of MHV68.

Summary and Description of the Invention:

According to an aspect of the present invention, M3 protein and its homologues can be used to bind chemokines of the immune system and their analogues, and to block binding of chemokines to corresponding cell surface receptors. Details of these binding effects of M3 protein are described herein below.

Homologues of M3 protein (e.g. obtained by mutation of an M3-encoding nucelotide sequence and expression from the mutated sequence, and/or by use or derivation from related gene sequences, e.g. from herpesvirus from Crocidura russula (Bowden, 1997, Cambridge University PhD thesis and Chastel et al, Acta Virologica 1994 38:309)), can be checked for their capacity to bind any or all of the chemokines mentioned below by appropriate equivalents of the cross-linking assays described herein, using for example radiolabelled chemokine.

The protein can for example be used to bind either chemokines and their

analogues with an animal species origin or specificity corresponding to the host range of the parent virus from which the protein comes, and/or chemokines and their analogues with human origin and/or specificity.

M3 protein can for example be used to bind C chemokines, CC chemokines, CXC chemokines or CX3C chemokines, for example the following: human lymphotactin (C chemokine); RANTES, MIP-1-alpha, MCP-1, MCP-4 (CC chemokines); IL-8, murine KC, murine MIP2, murine LIX, human GCP2, human IP10 (CXC chemokines); and fractalkine (CX3C chemokine).

In accordance with an aspect of the invention, M3 protein and its homologues can be used to inhibit the binding of such chemokines to their receptors, whether in-vitro, e.g. in biological samples, or in-vivo.

This effect can be exploited for example in specific binding tests using labelled reactants, e.g. for diagnostic and measurement purposes. The labelled reactant can be either the M3 protein, or the chemokine, or the chemokine receptor, according to the configuration of the test for desired purposes in hand.

The test configuration, and the corresponding form and composition of the reagents, can be selected from among known specific binding test configurations: e.g. ELISA tests; analogues of the original hormone radioimmunoassay configuration of Yalow and Berson, etc. Generally the test configuration involves contacting a biological sample with a labelled and/or immobilised form of a material with chemokine valency and/or a chemokine-binding agent, wherein the material with chemokine valency will normally be selected from chemokines as listed herein above, and compounds that can interfere with the binding of such a chemokine to its receptor, in order to detect or assay a substance with chemokine valency or its receptor possibly present in the sample.

Accordingly, an aspect of the invention also lies in compositions for carrying out such tests, e.g. the labelling product of M3 protein or a homologue; calibrated test aliquots of either of these; the product of binding M3 protein or a homologue to a solid phase suitable to take part in a specific binding test as

mentioned herein; calibrated test aliquots of one of the binding partners in the reaction; and test kits associating two or more of such reagents.

The test can be for example an assay for a chemokine or for a chemokine receptor. Examples of such tests can be arranged using variants of the binding test methods described in detail below.

The binding effect can also be exploited in the inhibition of effects mediated by chemokines that can be bound by the M3 protein or its homologues.

For example, it is known that in psoriasis IL8 is a mediator of pathological effects in skin. The binding effect described here can be used either in diagnostic methods to assess the degree of dependence of skin effects upon IL8 in a given case, or to produce a useful degree of inhibition of such effects.

In such a diagnostic method, sample material from skin tissue under test can be subjected to specific binding assay as indicated above to assess the presence and/or level of chemokine such as IL-8.

In an inhibitory treatment method, protein M3 or an analogue thereof can be applied to skin tissue either locally or systemically to modulate the interaction between chemokine and tis receptor in the tissue.

According to a further aspect of the invention a pharmaceutical composition can comprise M3 protein, or a homologue thereof as mentioned above, for use as an anti-inflammatory agent, in appropriate therapeutic (anti-inflammatory) amount.

According to a further aspect of the invention, a gene encoding M3 or a homologue thereof can be inserted under control of a suitable promoter, e.g. a strong tissue-specific or constitutive promoter such as the HCMV IE promoter, in a gene delivery system, e.g. for use in gene delivery in vivo. The gene delivery system can be a viral or non-viral vector system. Such a vector can be used to confer on a target transfected cell the ability to produce M3 protein or a

homologue thereof, e.g. for anti-inflammatory purposes when the target cell is in-vivo in a host that is the subject of treatment. Such anti-inflammatory purposes can include reduction of host immune response against elements of the vector delivery system and/or against other gene products expressed in the target cell after gene delivery by a vector system, whether it is from the same vector as that which delivers the M3 gene or from a separate delivery vector for such another delivered gene.

The invention, and materials and methods applicable to carrying out embodiments thereof, is further illustrated, but without intent to limit its scope, by the following description and accompanying drawings, which are described in further detail below, and of which:-

Figure 1 shows auto-radiographs of SDS-PAGE analysis, with molecular masses in kDa, from experiments in which soluble chemokine binding activity is produced by MHV68.

Figure 2 shows a further auto-radiograph of another SDS-PAGE analysis from an experiment to show binding specificity of the soluble chemokine binding protein encoded by the MHV68 M3 ORF.

Figure 3 is a graph showing binding of [125I] RANTES to test (U937) cells in the presence of different amounts of MHV68-infected cell supernatants expressed as cell equivalents.

Referring to the drawings, and to the descriptions of materials and methods given below:-

The inventors have verified, as described herein, that MHV68 encodes a soluble chemokine binding protein with broad specificity. In the first place, such activity has been detected in MHV68-infected cell culture supernatants. Analysis of the MHV68 genome has indicated that a unique ORF, M3, could be predicted to encode a secreted protein of around 40 kDa. The M3 ORF has been deleted from the MHV68 genome to check if the M3 ORF encodes the chemokine binding

activity. A revertant virus in which the M3 ORF was reinserted into the virus genome was also constructed to control for interactions elsewhere in the viral genome. The supernatants from wild type MHV68 and the M3 revertant infections formed a complex with [125I] RANTES after crosslinking, while the supernatants from the M3 deletion infection and the mock infection did not produce a complex (see Fig 1).

Figure 1 shows soluble chemokine binding activity produced by MHV68. For lanes 1-4, media from cultures uninfected (mock) or infected with MHV68 were incubated with [125I] RANTES and treated with the crosslinker BS3. The amount of medium used was equivalent to 5 x 10^2 cells. Lanes 5 and 6 media from mock baculovirus or baculovirus/M3 infected cells were incubated with [125I] IL-8 and treated with the crosslinker BS3. Auto-radiographs of the SDS-PAGE analysis, with molecular masses in kDa, are shown. The positions of RANTES (R), IL-8 and ligand-receptor complexes (square brackets) are indicated. Lane 1: MHV68 Wild Type, Lane 2: MHV68 M3 Revertant, Lane 3: MHV68 M3 Deletion, Lane 4: Mock, Lane 5: Baculovirus AcB15R, Lane 6: Baculovirus/M3.

Binding assays with [125I] IL-8 (CXC chemokine), [125I] RANTES and [125I] MIP-1α (CC chemokines) and [125I] Fractalkine (CX3C chemokine) were carried out with MHV68 infected cell supernatants followed by chemical crosslinking with BS3. Complexes were detected with all three classes of chemokine tested; the mock infections did not produce any complexes (Fig 1 and other data not shown in the Figures). The size of the complex in all cases was around 45 kDa, suggesting a MHV68 chemokine binding protein sized around 40 kDa after the size of the radiolabelled ligand was subtracted. Binding was detected whith a representative member of the CXC and CC and the single member of the CX3C subfamilies of chemokines indicating a broad binding specificity.

To further demonstrate that the protein encoded by M3 was capable of binding chemokines, a recombinant baculovirus was constructed which expressed the M3 ORF as protein. The supernatants from insect cells infected with this recombinant baculovirus/M3 was found to form a complex with [125I] IL-8 (Fig

1) of a similar size to that observed with MHV68 infected cell supernatants. Therefore the inventors conclude that the chemokine binding activity of the MHV68 infected cell supernatants is attributable to the product of the M3 ORF.

In order to determine the binding specificity of the product of the M3 ORF, crosslinking experiments were carried out with 2000 molar excess of unlabelled chemokine competitors. The binding to [125I] RANTES was competed to some extent by all the unlabelled chemokine competitors tested which included members from all four of the subfamilies (CXC, CC, C and CX3C) and examples of human and mouse chemokines (Fig 2). The different intensities of the bands suggested different affinities of the M3 protein for different chemokines. Further experiments indicated that M3 binding to IL-8 and MIP-1α could also be competed with Exodus-2 (also known as secondary lymphoid tissue chemokine SLC) as well as those chemokines listed in Fig 2.

Figure 2 shows binding specificity of the soluble chemokine binding protein encoded by the MHV68 M3 ORF, by crosslinking of 0.4 nM human [125I] RANTES with BS3 to medium from uninfected (mock) and infected cultures, in the absence (Lane 2) or in the presence of 2000-fold excess unlabelled chemokines from different species (Lanes 3-18). The amount of medium was equivalent to 5 x 10^2 infected cells. An auto-radiograph of the SDS-PAGE analysis showing the ligand-receptor complexes is shown. Lane 1: Mock infected cells, Lane 2: MHV68 infected cells uncompeted, Lane 3: Human RANTES, Lane 4: Mouse RANTES, Lane 5: Human MIP-1α, Lane 6: Mouse MIP-1α, Lane 7: Viral MIP-2, Lane 8: MCP-1, Lane 9: MCP-4, Lane 10: Murine KC, Lane 11: Human GROα, Lane 12: Human IL-8, Lane 13: Murine MIP-2, Lane 14: Murine LIX, Lane 15: Human GCP-2, Lane 16: IP-10, Lane 17: Human Lymphotactin, Lane 18: Fractalkine.

It has further been shown that the MHV68 soluble chemokine binding protein, M3, can block binding of chemokines to cell surface receptors.

Thus, a biological activity of M3 for CC chemokines was shown by the ability of supernatants from MHV68 infected cells to inhibit the binding of [125I]

RANTES to cellular receptors. The binding of [125I] RANTES to U937 cells was inhibited in a dose-dependent manner by MHV68 infected cell supernatants (Fig 3). These results indicated that the soluble protein encoded by the M3 ORF blocks the binding of CC chemokines to their high affinity cellular receptors. This was consistent with a high affinity interaction of RANTES and other CC chemokines with the M3 protein and strongly suggested that this viral chemokine binding protein is a potent inhibitor of the biological activity of chemokines, which is mediated by interaction with their cellular receptors.

Figure 3 illustrates binding of [125I] RANTES to U937 cells in the presence of different amounts of MHV68 infected cell supernatants expressed as cell equivalents. A single point is shown for mock infected supernatants (20000 cell equivalents) and a single point representing 100-fold excess of unlabelled RANTES. Means from duplicate samples are expressed as a percentages of counts binding in the absence of a competitor.

The fact that M3 blocks binding of chemokines to high-affinity chemokine receptors in U937 cells is regarded as an indication that the affinity of M3 for chemokines is similar to or better than that reported for the cellular chemokine receptors.

The inhibition of chemokine binding to cells as described herein can be achieved with very low doses of M3, which also gives an indication that it is a potent inhibitor of chemokine-receptor binding.

MATERIALS AND METHODS

Viruses and Infected Cell Supernatants

Viruses were grown and assayed on BHK 21 cells. Supernatants were prepared from BHK cells infected at 5 pfu per cell; the inoculum was removed after 2 hours and infected cells overlaid with Glasgow modified Eagles Medium (GMEM). Two days post infection the supernatants were collected, cellular debris removed by centrifugation and HEPES buffer (pH 7.5) was added to a final concentration of 20 mM. Supernatants were inactivated with 4,5',8-trimethylpsoralen and UV light (Tsung *et al.*, 1996).

Reagents

Radioiodinated recombinant human IL-8, RANTES and MIP-1α (2000 Ci/mmol) were obtained from Amersham (Little Chalfont, UK). Recombinant human RANTES was obtained from R&D Systems (Minneapolis, MN). Recombinant human macrophage inflammatory protein-1α (MIP-1α), viral MIP-2 from human herpesvirus-8, monocyte chemoattractant protein (MCP)-1, MCP-4, human interleukin-8 (IL-8), GRO-α, IFN-γ inducible protein 10 (IP-10), GCP2, lymphotactin, fractalkine and mouse RANTES, murine MIP-1α, murine KC, murine MIP2, and murine lipopoysaccharide-induced CXC chemokine (LIX) were obtained from PeproTech (Rocky Hill, NJ).

Binding Assays

Binding medium was RPMI 1640 containing 20 mM HEPES (pH 7.4) and 0.1% BSA. Crosslinking experiments with Bis(sulfosuccinimidyl) suberate (BS3) (50 μM) to [125I]-chemokines (0.4 nM) were performed in 25 μl as described (Alcamí & Smith, 1995, Upton *et al.*, 1992). Samples were analysed by 12% acrylamide SDS-PAGE gels. In the competition assays with U937 cells, supernatants were pre-incubated with 100 pM [125I] chemokine in 100 μl for 1 h at 4°C. Subsequently, 2.5 x 10^6 U937 cells were added in 50 μl and incubated for 2 h at 4°C. Bound [125I] chemokine was determined by phthalate oil centrifugation as described (Alcamí & Smith, 1992).

Construction of Recombinant Baculovirus

The MHV68 M3 ORF was amplified from infected cell DNA by PCR using oligonucleotides 5'-CGCGAATTCATGGCCTTCCTATCCACATCG-3' inserting an EcoRI site and 5'-GGTGCGGCCGCATGATCCCCAAAATACTCCAGC-3' which inserts a NotI site. The 1238 base pair product was digested with EcoRI and NotI and before being ligated in to EcoRI, NotI digested pBAC-1 (Novagen) creating pBACM3. The ORF was confirmed by sequencing before recombinant baculoviruses were produced as described (Alcamí & Smith, 1995). Recombinant M3 protein containing a C-terminal 6 histidine tag was produced in sf21 insect cells infected with recombinant baculovirus. The recombinant baculovirus AcB15R expressing the vaccinia virus soluble IL-1β receptor has been described

(Alcamí & Smith, 1992).

Further references cited in the methods section are as follows:-

Alcamí, A. & Smith, G. L. (1992). A soluble receptor for interleukin-1β encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. *Cell* **71**, 153-167.

Alcamí, A. & Smith, G. L. (1995). Vaccinia, cowpox and camelpox viruses encode interferon-γ receptors with novel broad species specificity. *J. Virol.* **69**, 4633-4639.

Tsung, K., Yim, J. H., Marti, W., Buller, R. M. L. & Norton, J. A. (1996). Gene expression and cytopathic effect of vaccinia virus inactivated by psoralen and long-wave UV light. *J. Virol.* **70**, 165-171.

Upton, C., Mossman, K. & McFadden, G. (1992). Encoding of a homolog of the IFN-gamma receptor by myxoma virus. *Science* **258**, 1369-1372.

The present invention also can be applied to the interaction of viruses such as human immunodeficiency virus and parasites such as Plasmodium vivax with attachment proteins on the surface of the target cells that they infect.

This application includes methods of inhibiting infection of susceptible cells by viruses such as human immunodeficiency virus and parasites such as Plasmodium vivax.

Since the M3 protein binds chemokines in solution and thus mimics the interaction of chemokines with cellular chemokine receptors, the M3 protein can also be expected to interact with the process of HIV or P. vivax infection.

In particular, it is expected that M3 protein can bind to attachment protein of HIV or Plasmodium vivax and thus prevent the binding of HIV or P. vivax to chemokine receptors in the host cell and/or subsequent entry into cells. M3 protein can thus be used to depress or prevent infection of target cells by these pathogens, and thus can protect or assist in protecting an individual from HIV or P. vivax infection. M3 protein can also be used to study the interaction of HIV and P.vivax with their target cells.

Chemokine receptors are known to play a critical role in transmission and dissemination of HIV by acting as a cofactor required together with CD4 for virus entry and infection (Faicu, 1996, Nature 384:529). The importance of

chemokine receptor CCR5 in vivo is evidenced by the finding that individuals who are homozygous for a mutant version of the CCR5 gene are resistant to HIV infection. Binding of chemokines or mutated chemokine antagonists to chemokine receptors can block HIV infection, illustrating the potential of the blockade of HIV-chemokine receptor interaction as a preventive and therapeutic strategy against HIV.

The malaria parasite Plasmodium vivax is known to use a chemokine receptor of unknown function (Duffy antigen) to enter and infect erythrocytes (Horuk et al., 1993, Science 261:1182). Similarly to HIV infections, binding of chemokines to the Duffy antigen blocks infection of erythrocytes by P. vivax, and individuals that lack the Duffy antigen on their red cells are resistant to P. vivax malaria. Thus, blockade of the interaction of the malaria parasite with Duffy antigen by M3 and homologues can be expected to be of use in connection with P.vivax infection, whether for intervention, investigation, or for development of drugs for P. vivax.

An inhibitor of virus or parasite interaction with chemokine receptor can be used to prevent or depress infection following transmission, e.g. in cases of accidental injection with HIV contaminated material. The M3 protein can also be used in combination with other anti-HIV therapies (none of them 100% effective).

The natural ligands of M3 are chemokines. Since gp120 of HIV is known to mimic chemokines and interact with chemokine receptors, M3 can be expected to interact with HIV gp120, particularly as it has such a broad binding specificity for chemokines as is shown herein. However, the affinity of M3 for gp120 may not be as high as it is for chemokines which are the natural ligand of M3.

According to an aspect of the invention, therefore, in-vitro DNA mutagenesis on the basis of the M3 gene can be used to create modified forms of M3, from which mutants can be selected that bind better to the gp120 of HIV (or to the attachment protein in P. vivax). (With the exception of a poxvirus 35K chemokine binding protein, M3 is the only soluble protein known to bind chemokines. So M3 protein can be used as a good starting point to develop such binding agents/inhibitors.

Thus, the M3 protein can be used as a starting material for mutation work with a view to deriving soluble proteins that can bind with higher affinity than M3 itself to the domain of HIV gp120 which interacts with the cellular chemokine

receptor, so as to facilitate blocking of HIV infection at an early stage. The M3 protein can also be used as a starting material for mutation work with a view to deriving agents to block attachment of P. vivax to the Duffy antigen on erythrocytes and initiation of infection.

The invention described and the disclosure made herein are susceptible to many modifications and variations as will be apparent to, and readily performable by, the skilled reader in the light of this disclosure; and the disclosure extends to adaptations, combinations and subcombinations of the features mentioned and/or described herein. Documents cited herein are hereby incorporated by reference in their entirety for all purposes.

Drawings 1/2

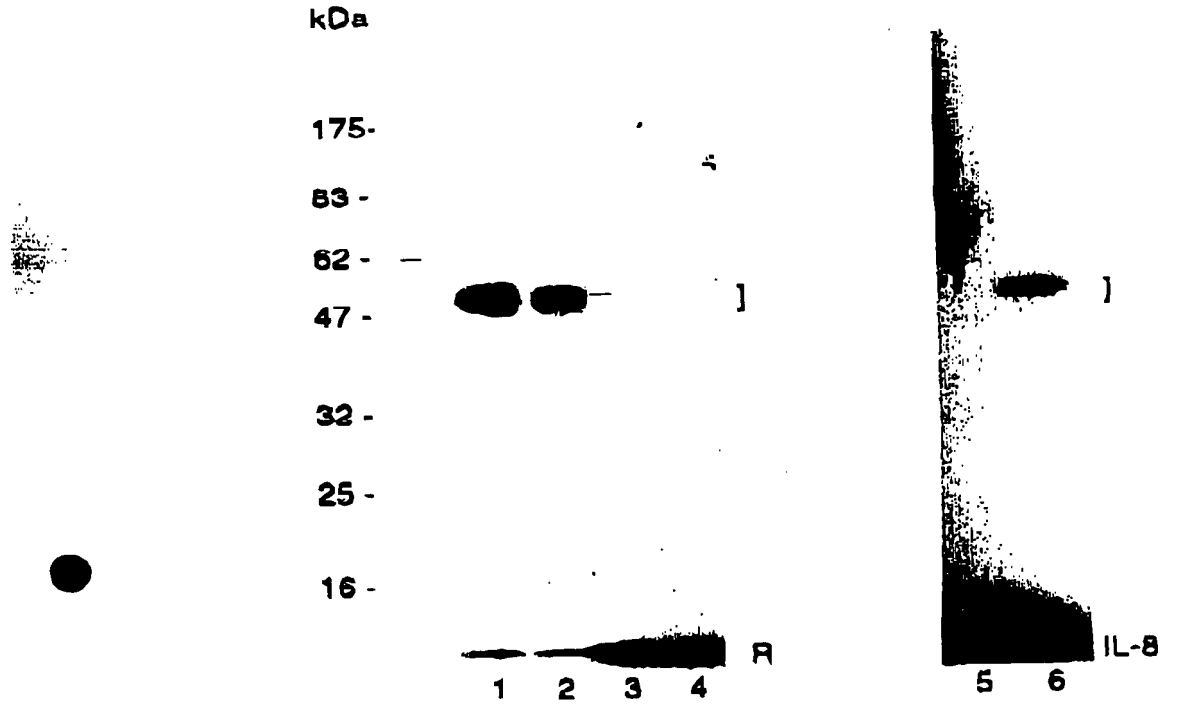


Figure 1

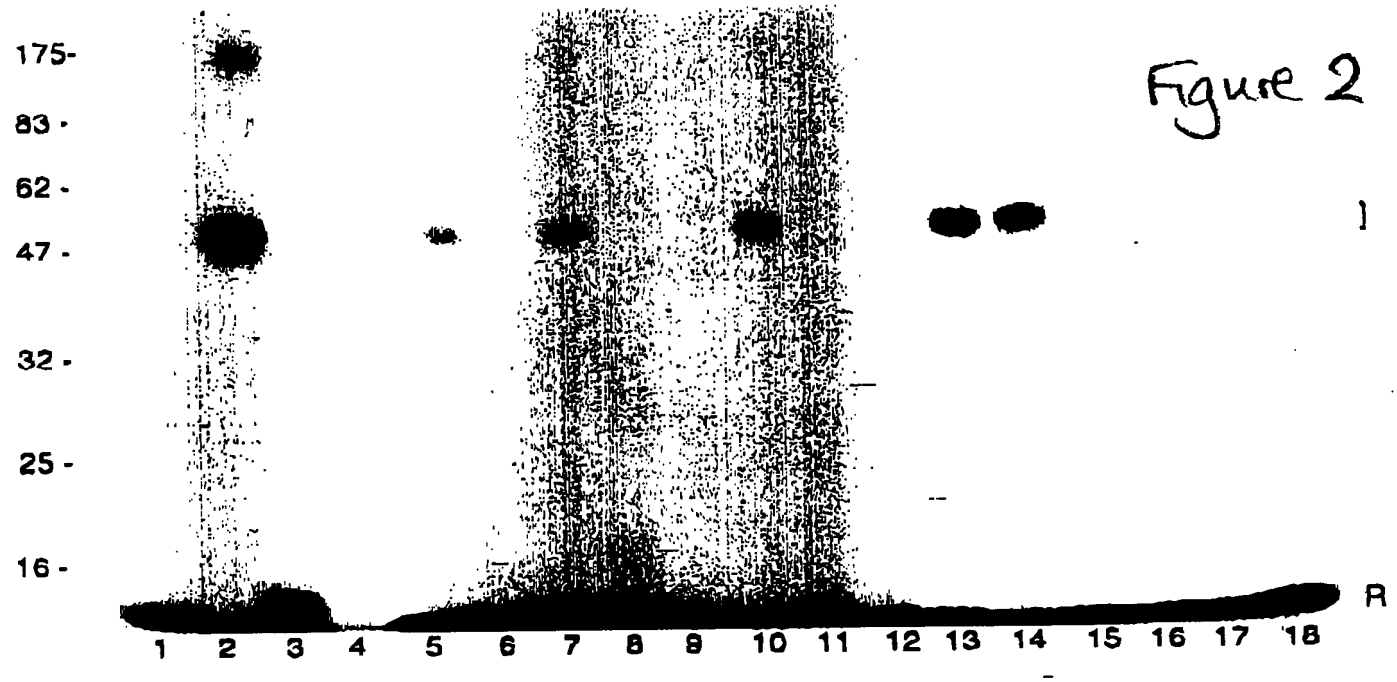
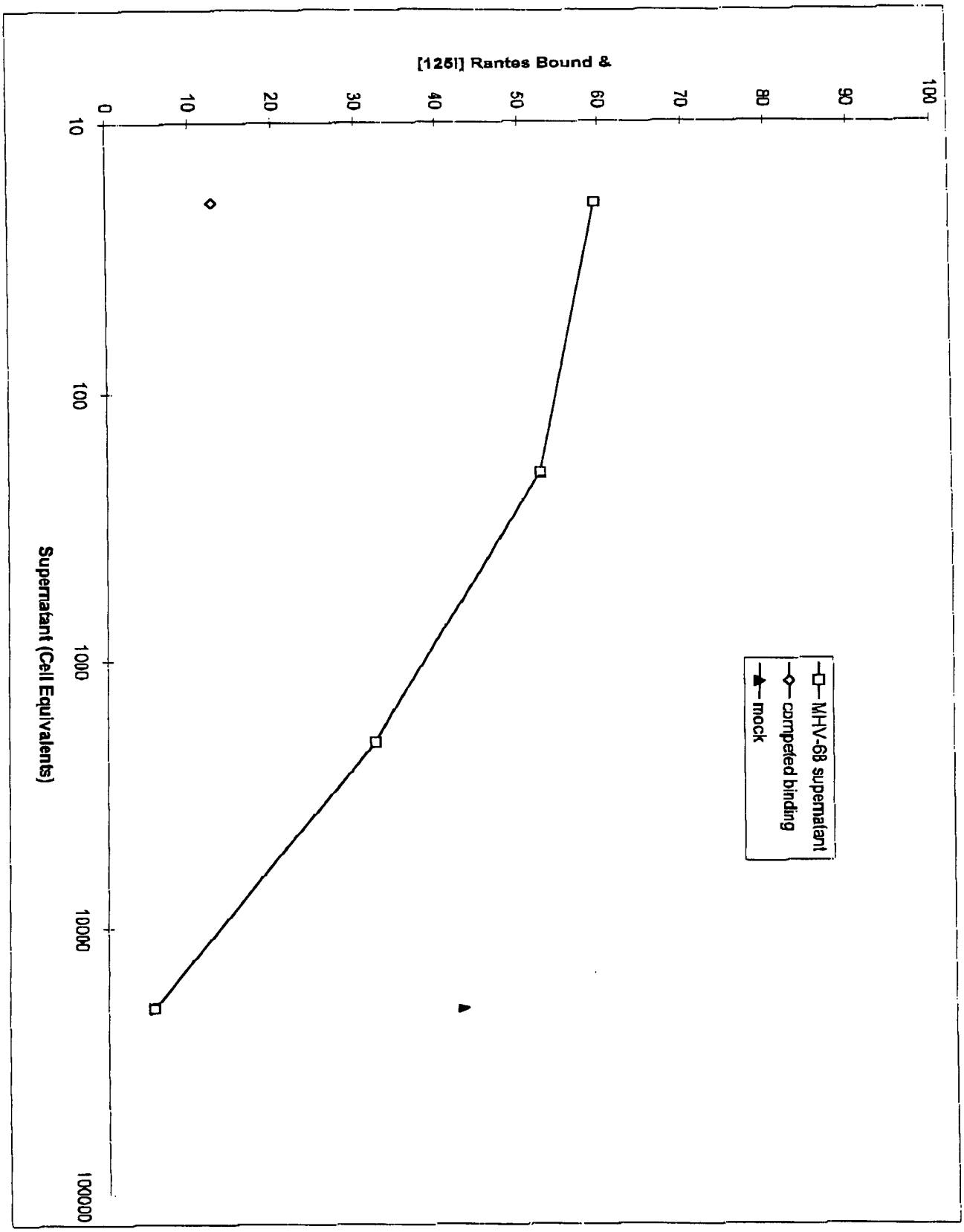


Figure 2

Figure 3


[125I] Rantes Bound &
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Supernatant (Cell Equivalents)
MHV-68 supernatant
competed binding
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